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Original research article

Prevalence and Genotypic Distribution of Hepatitis C Virus in Peshawar KPK, Pakistan

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ABSTRACT

This present study was planned to obtain an up-to-date picture of Hepatitis C virus (HCV) infection and its genotypes distribution in Peshawar, Khyber Pakhtunkhwa, Pakistan, as well as of the relationship between HCV genotypes and demographic and clinical parameters, and the risk factors in patients with an HCV subtype. Samples (blood) from 1978 individuals were collected and were tested using a strip-based method called the immunochromatographic test (ICT) for the existence of antibodies against HCV. It was observed that 158 of the 1978 individuals (7.9%) harbored antibodies in their blood against HCV, among which the female percentage (53.2%) was higher than that of the male (46.8%). Among the different age groups, the highest number of incidences of HCV antibodies was found in the age group of 31–40 years (26.6%). ICT positive samples were further screened by polymerase chain reaction (PCR) to determine the existence of active HCV-RNA, and it was found that 6.21% (123) of the total population (1978) tested, was positive, among which the female rate (56.91%) was observed to be higher than that of the male (43.09%). The highest incidence recorded was in the age group of 41–50 years (33.3%). HCV RNA positive individuals were genotyped: genotype 3a (45.5%) was dominant among the other detected genotypes, followed by 1a (11.4%), 3b (4.9%), and 2a (4.1%). It was concluded that the highest prevalence of HCV was found in females, and that the dominant genotype of the screened individuals was 3a genotype.

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1. Introduction

Hepatitis C is a chronic liver disease mainly caused by a diminutive (50 nm in size) enveloped, positive-sense, single-stranded RNA (ssRNA) virus called Hepatitis C virus (HCV). HCV is a member of the genus *hepacivirus*. It was first identified as a new viral agent causing non-A, non-B hepatitis (Chen and Morgan 2006; Choo *et al.* 1989). It is a major cause of liver dysfunction leading to liver injury, cirrhosis and hepatocellular carcinoma, and is a prominent health problem all over the world, especially in developing countries (Wedemeyer *et al.* 2014), such as Egypt and Pakistan (Averhoff *et al.* 2012). The principal

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route of transmission of HCV to the host mainly occurs through blood transfusion, un-sterilized surgical instruments, dental surgical instruments, the use of illegal drugs, the sharing of daily use things of an infected person, and acupuncture, while sexual transmission and interfamilial transmission have been also reported (Qureshi *et al.* 2013). Although symptoms may be not severe for decades, approximately 20% of infected patients may suffer from lethal liver injury and liver cancer (Imran *et al.* 2013). Recently, a report on HCV infection in Pakistan was published in 2007–2008 by the Pakistan Medical Research Council. A total of about 47,000 individuals from the all provinces of Pakistan were screened, and the anti-HCV was found to be present in 4.8% of the population (Dhingra *et al.* 2014). In view of the fact that the HCV genome is highly heterogeneous in nature, to date it has been grouped into at least six major genotypes (genotypes 1–6), which can be further classified into various small sub-groups world-wide (Gower *et al.* 2014). Of

note, the classification of the HCV genotype into groups and subgroups plays a key role in the study of geographical and demographic differences, as well as in molecular epidemiology, vaccine development, therapeutic decision-making, and the study of chronic HCV infection. Hence, it provides important clinical information for health improvement planning and policy. The prevalence of HCV is slightly higher than in developed countries because of the limited availability of resources (Gul *et al.* 2016). Although there is a need among the large populations of the developing world to study the relationship of HCV by genotype with age, gender, route of entry and the duration of infection, there is currently a lack of relevant data available. The aim of this present study was therefore to obtain an up-to-date picture of the distribution of HCV infection and its genotypes in Peshawar city, Khyber Pakhtunkhwa, Pakistan, as well as to analyze the relationship between HCV genotypes and demographic and clinical parameters, and to look at the risk factors present in patients with an HCV subtype.

The hope is that this present study will also help to draw the attention of local and foreign organizations to this important issue. Peshawar city was decided on as a good site to conduct the research because of the very low literacy rate in almost all of its districts, the limited availability of health facilities, and the frequency of natural disasters such as floods. The polymerase chain reaction (PCR) method holds an advantage over non-PCR immunoassays such as enzyme-linked immunosorbent assay (ELISA), recombinant immunoblot assay (RIBA), enzyme linked immunosorbent assay (EIA), immunochromatographic test (ICT), and so on, being a quick and reliable tool to diagnose, genotype and quantify active HCV RNA in the blood. However, to date, there is no PCR-based prevalence studies with large sample numbers available for the population of Pakistan.

2. Materials and Methods

2.1. Sampling

This study was conducted in the Alfalah PCR Laboratory and Research Center, Dabgari Garden capital province Peshawar, Khyber Pakhtunkhwa, Pakistan from February 2013 to January 2015, in which blood samples of 1978 individuals comprising 809 males and 1169 females were taken through sera and extracted. All necessary information were gained through proper questionnaires, informed consent and ethical approvals were taken from each infected patient as directed by expert physician and owner of the hospital, and kept confidential. All the individuals were categorized into age groups 10–20, 21–30, 31–40, 41–50 and over 50 years, respectively.

2.2. Initial screening

Initially, the sera were screened for the presence of anti-HCV antibodies using a strip-based method called an ICT. For this purpose, a commercially available kit (ACON®, ACON Laboratories Inc., San Diego, CA, USA) was used according to the protocol recommended by the manufacturer. The positive samples were further processed for molecular detection and genotyping.

2.3. PCR confirmation of HCV

From the samples that had tested positive for anti-HCV antibodies, HCV RNA was extracted using a TRIzol reagent (Invitrogen, Carlsbad, USA) following the protocol recommended by the company. The complementary DNA (cDNA) was synthesized using gene-specific reverse primers by reverse transcription, using Moloney murine leukemia virus reverse transcriptase at 37°C for 50 minutes. The viral RNA was confirmed through PCR using primers

whereas the primers used for internal core region amplification were:

Forward: 5'-GAAAGCGTCTAGCCATGGCG-3' and
Reverse: 5'-CACAAAGGCCTTCGCGACC-3'.

2.4. PCR-based identification of HCV strains

RNA from each sample was isolated by following the Ohno *et al.* (1997) protocol. Reverse transcription PCR was performed for viral cDNA generation using core gene-specific reverse primers, and multiplex-PCR was performed for genotypes identification. Two reaction mixtures, containing two sets of primers each, were processed in parallel in the nested PCR (Table 1). The PCR-thermal cycler was set to 94°C for 5 minutes for pre-denaturation, followed by denaturation at 94°C for 30 seconds (30 cycles). Primer annealing was carried out at 64°C for 30 seconds; this was followed by an extension at 72°C for 30 seconds, and a final temperature of 4°C for 10 minutes. The same conditions were applied for the nested PCR; however, primer annealing was carried out at 53°C.

3. Results

3.1. Initial screening

A total of 1978 individuals consisting of 809 males and 1169 females were initially screened using ICT for the presence of antibodies against HCV. The results showed that 158 of the 1978 individuals (7.9%) were positive. Among the positive individuals, 74 (46.8%) were males and 84 (53.2%) were females (Figure 1).

3.2. PCR-based confirmation of active HCV

The detection of antibodies against HCV is not a reliable test for HCV. Therefore, all anti-HCV carrying samples were confirmed for the presence of HCV RNA using PCR (Figure 2). It was observed that 123 individuals (77.8%) tested positive for the presence of HCV RNA. More incidences were observed in females (70; 56.91%) as compared with males (53; 43.09%) (Figure 1).

Among the different age groups, the highest incidence of anti-HCV antibodies (42; 26.6%) was observed in the following age groups: 31–40 years, followed by the 21–30 years (35; 22.2%), 51

Table 1. Details of primers used for genotyping, as described by Ohno *et al.* (1997)

Primer	Sequence (5'–3')	Expected band size (bp)
Sc2	GGGAGGTCTCGTAGACCGTGACCATG	441
Ac2	GAG(AC)GG(GT)AT(AG)TACCCCATGAG(AG)TCGGC	
Mix1 primer		
S7	AGACCGTGACCATGAGCAC	
S2a	AACACTAACCGTCGCCACAA	
G1b	CCTGCCCTCGGGTTGGCTA(AG)	234
G2a	CACGTGGCTGGGATCGCTCC	139&190
G2b	GGCCCCAATTAGGACGAGAC	337
G3b	CGCTCGGAAGTCTTACGTAC	176
Mix2 primer		
S7	AGACCGTGACCATGAGCAC	
G1a	GGATAGGCTGACGTCTACCT	208
G3a	GCCCAGGACCGGCTTCGCT	232
G4	CCCGGAACCTAACGTCCAT	99
G5a	GAACCTCGGGGGAGAGCAA	320
G6a	GGTCATTGGGGCCCAATGT	346

For the naming of primers, S = sense; A or G = antisense and C = core region; the notations 1a–6a are in accordance with the HCV genotype nomenclature proposed by Simmonds *et al.* (1994). Numbering is from the authentic start codon of the open reading frame.

2s, second-round PCR for sequencing; 2g, second-round PCR for genotyping. Pairs of nucleotides inside parentheses denote degenerate nucleotides sequence.

(F): 5'-CCCTGTGAGGAAGTACTGTCTTCACGC-3', and
(R): 5'-ACTCGCAAGCACCCTATCAGGCAGTAC-3',

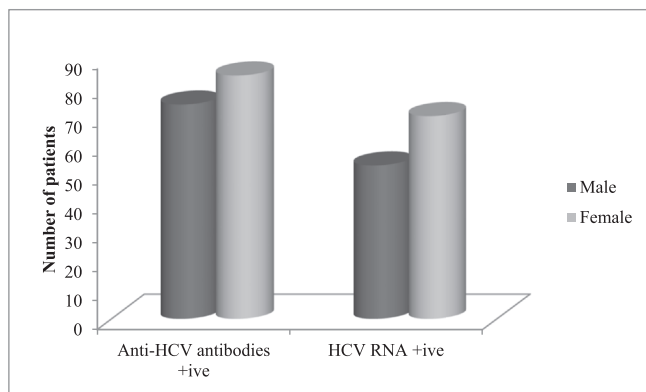


Figure 1. Sex-wise distribution of the anti-HCV antibodies and HCV RNA. HCV = Hepatitis C virus.

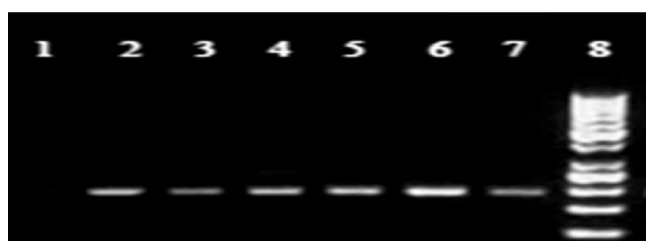


Figure 2. Representative gel photograph of HCV amplified products. Lane 1 is negative control, lanes 2–7 are the typical positive samples for active HCV showing expecting 220 bp band of hepatitis C virus and lane 8 represents molecular marker (1 kb). HCV = Hepatitis C virus.

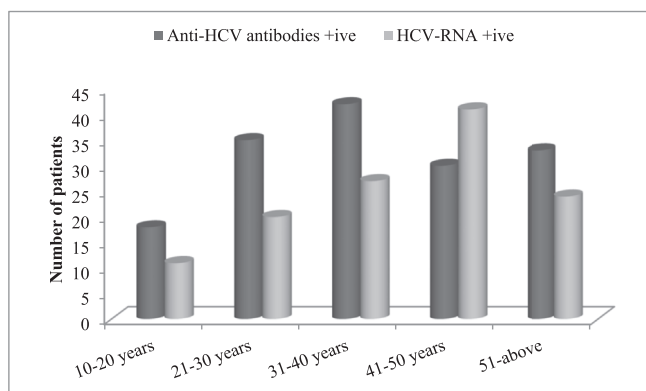


Figure 3. Age-wise distribution of the anti-HCV antibodies and HCV RNA positive. HCV = Hepatitis C virus.

years and above (33; 20.9%), 41–50 years (30; 19%) and 10–20 years (18; 11.4%) (Figure 3). Similarly, the highest incidence of the presence of HCV RNA was observed in the following age groups: 41–50 years (41; 33.3%), followed by 31–40 years (27; 22.0%), 51 years and above (24; 19.5%), 21–30 years (20; 16.3%) and 10–20 years (11; 8.9%) (Figure 3).

3.3. Distribution of HCV genotypes among different age groups

The HCV RNA positive samples (123) were genotyped. Among the samples tested, genotype 3a was found to be the most dominant genotype, being detected in 56 of 123 individuals (45.5%) among all the age groups. The second most dominant viral

genotype was 1a, which was detected in 14 individuals (11.4%), followed by 3b and 2a which were detected in 6 (4.9%) and 5 (4.1%) individuals, respectively. A total of 22 samples (17.9%) were found with untypeable genotypes, as no genotype-specific PCR fragments band was observed, whereas the infection of two (mixed) different genotypes was observed in 20 individuals (16.3%) (Figure 4).

4. Discussion

Viral hepatitis is alarmingly high among the general population of Pakistan; ten million people or 6% of the total population of Pakistan is infected with HCV (Raja and Janjua 2008). In this study, a random sample was taken from the mixed population of Peshawar city, Pakistan, including the majority Pashtun, other smaller ethnic groups such as the Hindkows, Dards, Chitralis and Gujjars, and Afghan refugees. The molecular epidemiology of HCV varies by region and in different groups of the same population (Idrees *et al.* 2008, Wild and Hall 2000). In our study, 6.21% prevalence of HCV was observed, which is comparatively higher than that of Islamabad (5.31%) (Idrees *et al.* 2008), Rawalpindi (2.45%) (Masood *et al.* 2007), Bunir (5%) (Muhammad and Jan 2005), Multan (4.06%) (Idrees *et al.* 2008), Karachi (4%–6%) (Khan *et al.* 2011), and lower than that of Faisalabad (20.89%) (Ahmad *et al.* 2007), Mardan (9%) (Ali *et al.* 2010) and Northern Areas (25.7%) (Tariq *et al.* 1999). This could be due to various factors including the lack of quality health care, poor literacy rate, low average socio-economic status, general lack of awareness, lack of availability of suitable screening facilities, and differences in population samples selected for the study (Shah *et al.* 1997). When comparing the different age groups, we found that the highest prevalence of HCV infection occurred among adult men. Age has been argued to be a major factor in HCV studies, with infection more predominant in older persons (Cozzolongo *et al.* 2009, Mengal *et al.* 2012). Our study clearly indicates the dominance of genotypes 3a (45.5%) and 1a (11.4%) among the tested population. These results validate the findings of Ahmad *et al.* (2010), who reported that 59.9% and 16.5% of individuals were infected with 3a and 1a genotypes, respectively, both these being higher percentages than the other genotypes detected. Many other studies have also reported that genotype 3a occurs most frequently in Pakistan (Afridi *et al.* 2014, Ahmad *et al.* 2010). Genotype 4a was not detected in our study; this genotype seems to be very rare in Pakistan. However, Ahmad *et al.* (2010) isolated genotype 4a in a study of prevalence in Lahore, Pakistan. Other studies reveal that genotype 4a is prevalent in the Middle East (Abdulkarim *et al.* 1998, Gower *et al.* 2014). Genotypes 5a and 6a were also not isolated in our study. Acquaintance about the occurrence of different HCV genotypes in different regions of Pakistan is

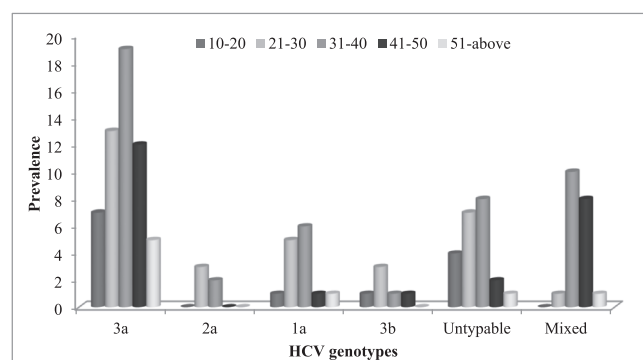


Figure 4. Distribution of HCV genotypes in Peshawar province. HCV = Hepatitis C virus.

considered to be an important clinical parameter for molecular epidemiology, vaccine development, therapeutic implications and chronic HCV infection (Ahmad *et al.* 2010, Ali *et al.* 2010). HCV is reported to exhibit elevated levels of genetic variation. On the basis of nucleotide sequences recuperated from HCV infected persons, HCV is categorized into six genotypes and several subtypes (Smith *et al.* 2014). These genotypes showed 30%–35% nucleotide differences in their genomes. However, these differences are not evenly spread throughout the genome, as a very large variability is present within the viral glycoproteins (Simmonds 2004). HCV genotypes show a discrepancy in distribution. Genotypes 1–3 are very widely distributed, whereas genotype 4 is more prominent in Africa and the Middle East, genotype 5 is prominent in South Africa, genotype 6 in South East Asia and genotype 7 in Central Africa (Gower *et al.* 2014, Simmonds 2004).

In our study, an unknown HCV genotype was detected in 27 of 123 individuals (17.9%). Most HCV studies in Pakistan reveal the isolation and detection of an unknown/undetermined HCV genotype (Ahmad *et al.* 2010, Ali *et al.* 2010, Rauf *et al.* 2011). In future, a significant challenge for researchers and experts is the identification of this/these unknown genotype(s), and the exploration of its/their possible role in the development of Hepatitis C. This will help to ease clinical management, the process of diagnosis, vaccine development and other prevention measures.

Conflict of interest

There is no conflict of interest.

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